Available online at www.sciencedirect.com

ScienceDirect

journal homepage: http://www.elsevier.com/locate/euprot

Evolutionary conservation of the mature oocyte proteome

Tamar Lotan[a,*], *Vered Chalifa-Caspi*[b], *Tamar Ziv*[c], *Vera Brekhman*[a], *Michal Markovich Gordon*[b], *Arie Admon*[c], *Esther Lubzens*[c,**]

[a] *Marine Biology Department, The Leon H. Charney School of Marine Sciences, University of Haifa, Haifa 31905, Israel*
[b] *Bioinformatics Core Facility, National Institute for Biotechnology in the Negev, Ben-Gurion University of the Negev, Beer-Sheva 84105, Israel*
[c] *Faculty of Biology, Technion – Israel Institute of Technology, Haifa 32000, Israel*

ARTICLE INFO

Article history:
Received 25 July 2013
Received in revised form
26 November 2013
Accepted 29 January 2014
Available online 15 February 2014

Keywords:
Oocyte
Proteomics
Evolution
Vitellogenin
Germ cell

ABSTRACT

The proteome profiles of mature ovulated oocytes of the Cnidaria basal eumetazoan, the starlet sea anemone *Nematostella vectensis* was compared with published data of mammalian mouse mature oocytes. We identified 1837 proteins in *N. vectensis* oocytes including known oocyte- and germ-cell-specific markers, proteins associated with RNPs and vitellogenin, a major component of egg yolk proteins. Our findings suggest highly conserved enriched functional pathways in *N. vectensis* and the mouse mature oocytes. This study provides the first catalog of cnidarian oocyte proteins, revealing highly conserved ancient organization of life processes for over 500 million years of evolution.

Significance: The current study provides the first proteomic profile of an oocyte of a cnidarian organism the starlet sea anemone *N. vectensis* and gives new insights on the ancient origin of an oocyte proteome template. The comparative analysis with a chordate oocyte suggests that the oocyte proteome predates the divergence of the cnidarian and bilaterian lineages. In addition, the data generated in the study will serve as a valuable resource for further developmental and evolutional studies.

1. Introduction

A mature oocyte contains the full complement of maternal proteins and mRNAs required for fertilization and subsequent embryonic development. Because oocytes and early embryos are transcriptionally silent, developmental processes at these stages rely exclusively on maternal mRNAs and proteins [1–3]. Substantial research efforts have been devoted to unraveling of the molecular pathways governing gene expression and genome reprogramming and their contribution to cell differentiation, pluripotency and germ-cell formation in oocytes of eumetazoans [4,5]. Those studies pointed to conservation of functions as numerous genes expressed in oocytes were found to be conserved in oocytes of mouse, bovids, *Xenopus laevis* and *Ciona intestinalis* [4,5]. Comparative proteome profiling studies are still lacking at this stage, although information is available on proteome profiles of model and non-model

* *Corresponding author*. Tel.: +972 4 8240034.
** *Corresponding author*. Tel.: +972 77 8871903.
E-mail addresses: lotant@univ.haifa.ac.il (T. Lotan), elubzens@tx.technion.ac.il (E. Lubzens).
http://dx.doi.org/10.1016/j.euprot.2014.01.003

organisms. These include oocyte proteomes of *Caenorhabditis elegans* [6], sea urchin [7], zebrafish [8,9] and the most intensively studied mouse [10–13]. Proteomic profiling offers a novel insight into facets of oocyte function which, owing to discrepancies between protein levels and the levels of corresponding transcripts, have not yet been revealed [14]. Such divergence between transcriptome and proteomic profiles in oocytes is particularly expected as oocytes contain translational silent mRNAs that are activated only after fertilization [3]. Moreover, oocytes of most oviparous eumetazoans accumulate yolk proteins that are not synthesized by the oocyte itself. In the present study we investigated evolutionarily conserved maternal proteins in oocytes by comparing the *Nematostella vectensis* oocyte proteomic profile with the published proteomic profiles of mouse oocytes at the metaphase II (MII) stage [11,13].

There is growing interest in cnidarians as representatives of ancestral basic eumetazoans because of their potential of offering important insights into early evolution [15]. As basal eumetazoans, diploblastic cnidarians form an outgroup of the bilaterian animals and are intermediate in complexity between sponges and bilaterians. An emerging developmental model system of cnidarians is the starlet sea anemone *N. vectensis* which has a published genome [16] and whose sexual reproduction can be induced and controlled in the laboratory. *N. vectensis* produce eggs whose ultrastructure during oogenesis exhibits features in common with other oviparous eumetazoans [17]. This raises the question of whether proteins present in this basal diploblastic model species have been retained through evolution suggesting conservation of oocyte-specific proteins. The expression of critical genes in bilaterian germ-cell specifications and genes that represent canonical pathways in bilaterian embryonic developmental have been described in *N. vectensis* [15,18].

In this study we generated the first catalog of cnidarian oocyte proteins of *N. vectensis* ovulated eggs. Ovulated oocytes, or eggs, are arrested at MII and are normally fertilized within a few hours. The terms “oocyte” and “egg” are used quite differently in various species, as mature ovulated oocytes in oviparous species are usually called “eggs”, whereas “oocytes” is the corresponding term in mammals. We identified 1837 proteins in mature ovulated oocytes of *N. vectensis* and show their putative similarity to oocyte of MII mouse (*Mus musculus*). Some of the identified proteins were associated with oocyte structure and function, while others were germ-cell-specific. We have gained fundamental insight into the proteome of oviparous *N. vectensis* eggs and highlighted putative proteins and functions in a basal eumetazoan that are shared with or differ from mammalian MII oocytes.

2. Materials and methods

2.1. Cultures of N. vectensis

N. vectensis were cultured in 12.5 ppt Red Sea salts at 18 °C and spawning was induced as previously described [19].

2.2. Collection, extraction and proteolysis of oocytes

Mature oocytes were isolated from their gelatinous egg sack using 3% cysteine (Fig. 1) [20] and washed in excess culture medium over 50 μ nylon mesh. Biological triplicates of 20 eggs (0.4 μg per oocyte) were collected from three different anemone groups and each sample contained mature oocytes from 4 to 6 egg sacks. The oocytes were extracted in 8 M urea, 400 mM ammonium bicarbonate and 10 mM DTT, and sonicated. 20 μg protein from each sample were reduced with 2.8 mM DTT (60 °C for 30 min), modified with 8.8 mM iodoacetamide in 400 mM ammonium bicarbonate (in the dark, room temperature for 30 min) and digested in 2 M urea, 25 mM ammonium bicarbonate with modified trypsin (Promega) at a 1:50 enzyme-to-substrate ratio, overnight at 37 °C. An additional second trypsinization was done for 4 h.

2.3. Mass spectrometry analysis

The tryptic peptides were desalted using C18 tips (Harvard Apparatus) dried and re-suspended in 0.1% formic acid. The peptides were resolved by reversed-phase chromatography on 0.075 × 200-mm homemade fused silica capillaries (J&W) packed with Reprosil reversed phase material (Dr. Maisch GmbH, Germany). The peptides were eluted with linear 214 min gradients of 5–35% and 8 min at 95% acetonitrile with 0.1% formic acid in water, at flow rates of 0.15 μl/min and the LC system was EASY nLC 1000 (Thermo). Mass spectrometry was performed by Q-Exactive mass spectrometer (Thermo-Fisher) in positive mode using repetitively full MS scan followed by higher-energy collisional dissociation (HCD) of the 10 most dominant ions selected from the first full MS scan. The mass spectrometry data from three biological repeats was analyzed using the MaxQuant software 1.3.0.5 [21] vs. the Nematostella section of the Uniprot database with 1% FDR (see below). The data was quantified by label free analysis using the same software. Label free analysis compares intensities across runs. Briefly, for every peptide, corresponding total signals from multiple runs were compared to determine peptide ratios. Pair-wise peptide ratios were only determined when the corresponding peak is detected in both LC–MS runs. A robust estimation of the protein ratio is calculated as the median of pair-wise peptide ratios interpolated with the square root of the ratio of summed-up intensities. The analysis is done after first applying a normalization procedure and recalibration of the retention times [21].

2.4. Bioinformatics analysis

2.4.1. Analysis of N. vectensis data

N. vectensis protein sequences and annotations, as well as their InterPro and PFAM domains, were retrieved from the UniProt database (date 25.2.2013). The dataset contained 24,999 proteins, out of which 1202 proteins had a meaningful annotation (i.e. not “predicted protein”). The sequences were submitted to the Blast2Go software, where blastp was run against the NCBI RefSeq protein database. A maximum of 20 hits with e-value $< 10^{-3}$ were retrieved for each query sequence. The annotations obtained from Uniprot and Blast2GO were used to construct a FASTA file which then served as a reference proteome in the analysis of the mass spectrometer data. The expressed proteins were tested

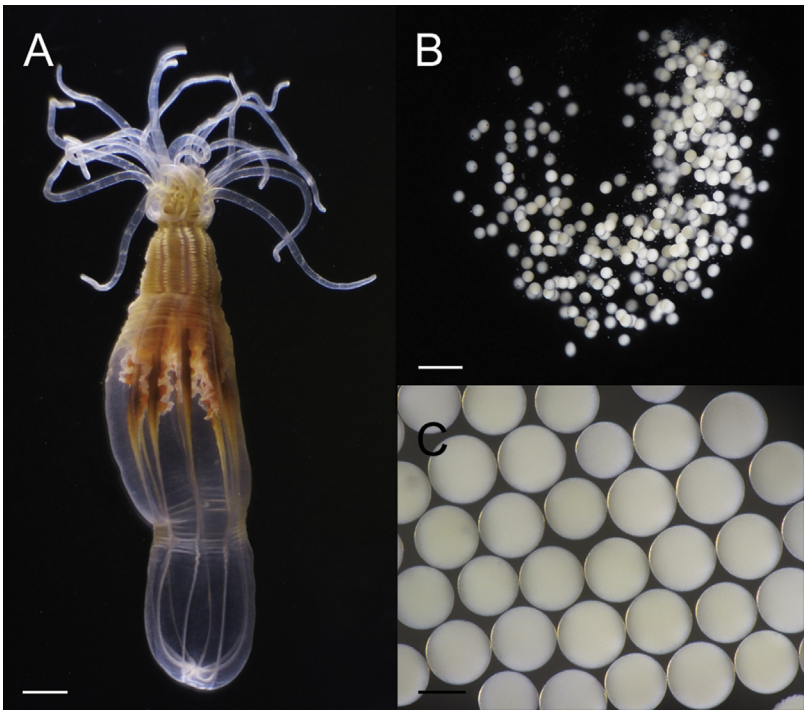

Fig. 1 – Photographs of reproductive *N. vectensis*. (A) A mature *N. vectensis* female; bar – 1 mm. (B) Secreted gelatinous egg sac; bar – 500 μ. (C) Isolated oocytes; bar – 100 μ.

for enrichment against GO biological processes and KEGG pathways using Blast2GO and KOBAS [22], respectively. In both cases, enrichment was tested using Fisher exact test (FDR < 0.05).

2.4.2. *Comparison with the mouse MII oocytes proteome*

International Protein Index (IPI) IDs of proteins expressed in mouse oocytes at the MII stage were extracted from the supplementary file sd01.xls of Wang et al. [13] and from Supplement Table 1 of Pfeiffer et al. [11]. These IPI IDs were converted to Uniprot IDs. In order to compare the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways expressed in the mouse MII oocytes and the *N. vectensis* mature oocytes, the UniProt IDs of the expressed proteins from each organism were assigned with universal KEGG Orthology (KO) IDs through parsing of UniProt entries. The relation of KO IDs to KEGG pathways was retrieved through KEGG API (http://www.kegg.jp/kegg/rest/). Visualization of expressed proteins on the KEGG pathway maps was achieved through the "KEGG Mapper – Search & Color Pathway" tool. Pathway enrichment of the mouse proteins was done using KOBAS (http://kobas.cbi.pku.edu.cn/home.do). To identify orthologous proteins between *N. vectensis* and mouse we used reciprocal blastp analyses (NCBI-BLAST version 2.2.25) of *N. vectensis* against mouse and vice versa, using an *e*-value cutoff of $10e^{-5}$ and the effective length of the database was set to 169,539,820. This was done to eliminate the dependency of the *e*-values on the proteome sizes, thus obtaining comparable *e*-values for the two (reciprocal) comparisons.

3. Results

3.1. *Proteomic profiling of mature oocytes of N. vectensis*

We identified 1412 protein groups consisting of 1837 proteins by μLC–MS/MS analysis using a Q-Exactive LTQ-Orbitrap mass spectrometer. Several protein groups consisted of more than one protein, displaying identical peptide sequences. The complete list of proteins and protein groups of mature ovulated oocytes from *N. vectensis* is presented in Table S1. Most of the proteins were annotated with Blast2GO, but the functions of 134 proteins in 95 protein groups remain unknown. Of the total protein groups, 58% (821 proteins) could be matched to known KEGG functions (with a KEGG Orthology, KO number) (Table S2). We compared the *N. vectensis* mature oocyte proteins to those of MTII mouse listed in two recent publications [11,13] containing 2973 and 3699 proteins, respectively, and a total of 4425 unique groups, of which 2368 could be assigned a KO number. We found that 84% (690) proteins of *N. vectensis* assigned KO number were also found in MII oocytes and 16% (130) proteins were unique to *N. vectensis* (Table S2). It should be noted that the identified protein repertoire of *N. vectensis* is smaller than the profiles for MII mouse oocytes. Oocyte proteins may have not been detected due to technical limitations or because of insufficiency in the annotation of the *N. vectensis* genome.

Vitellogenin (Vtg) constitutes the major protein component of *N. vectensis* oocytes (about 67%) (Fig. 2A). The other 25 most abundant protein groups consist mainly of

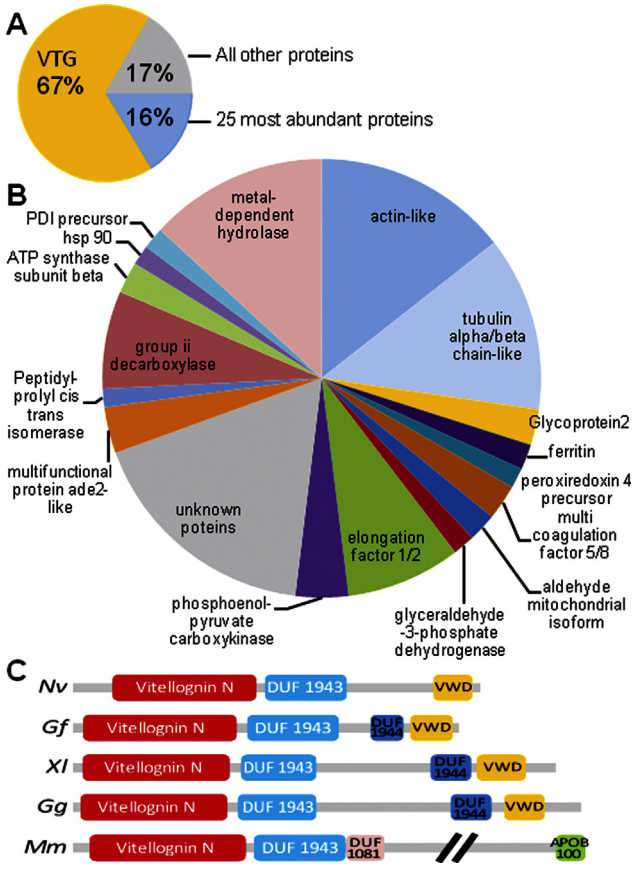

Fig. 2 – Most abundant *N. vectensis* ovulated oocyte proteins. (A) Vtg and the 25 most abundant proteins relative to all proteins identified in the oocyte. (B) The 25 most abundant protein groups in the oocyte (without Vtg). (C) Schematic diagram comparing the Vtg of *N. vectensis* (*Nv*) (A7S177), with Vtg's of the coral *Galaxea fascicularis* (*Gf*) (Q5R227), *Xenopus laevis* (*Xl*) (Q7SZF6), Chicken *Gallus gallus* (*Gg*) (Q5R227) and the apolipoprotein B of *Mus muscullus* (*Mm*) (E9Q414). Vtg's domains are: lipid transport proteins N terminal (Vitellogenin_N), several domains of unknown function as DUF1943, DUF1944 and DUF1081, the von Willebrand factor type D (vWD) and apolipoprotein B100 (APOB100), a domain found in mouse apolipoprotein B protein.

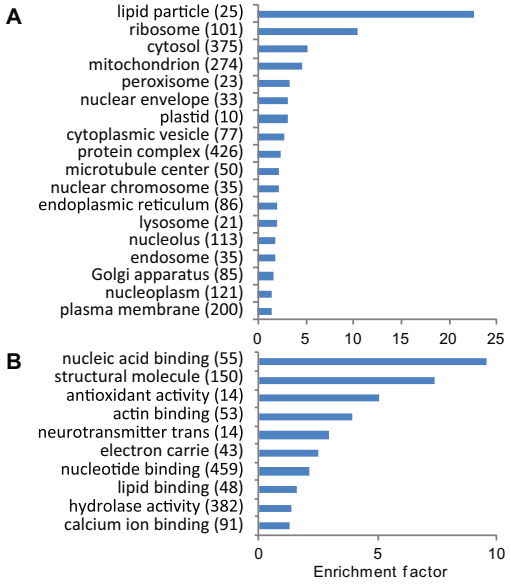

Fig. 3 – Gene ontology (GO) categorization of the identified oocyte proteins. (A) Cellular function. (B) Molecular processes. The enrichment factors of GO terms that are significantly overrepresented (FDR < 0.05) in the oocyte with respect to all *N. vectensis* proteins are illustrated. The number of proteins in each GO term is indicated in brackets.

cytoskeletal proteins such as actin and tubulin and enzymes such as decarboxylases, ATP-synthase, and elongation factors. Within this group of the most abundant proteins, nine proteins are unknown (Fig. 2B). Gene ontology (GO) of cellular function shows that lipid particles and ribosomes are highly represented in the mature oocyte whereas for molecular processes GO shows an abundance of translational machinery, structural molecular activity, antioxidant activity, generation of precursor metabolism and energy, cytoplasmic organization and protein transport (Fig. 3).

3.2. *Vtg is the main oocyte yolk protein*

The *N. vectensis* Vtg protein (A7S177) shows conserved domain structure with Vtgs of other vertebrate and invertebrate species (Fig. 2C). It consists of the Large Lipid Transfer module (LLT/Vitellogenin_N), DUF1943 and the von Willebrand factor type D (vWD) superfamily domains, but lacks DUF1944 and DUF1081 domains [23] of unknown functions. Although mammals do not display Vtg as their storage proteins LLT/Vitellogenin_N superfamily is present in lipid transport proteins as apolipoprotein B (Fig. 2C) and the Microsomal Triglyceride Transfer protein. Only one sequence in the *N. vectensis* genome can be matched with the oocyte Vtg protein; the previously suggested putative fragmented Vtg-like (A7S177) [23] was not found at the protein level. The site of Vtg synthesis in *N. vectensis* has yet to be determined, but the identification of vigilin (A7SFJ6), a high-density RNA-binding protein in *N. vectensis* oocytes suggests regulation of Vtg mRNA within the oocytes. Vigilin binds specifically to the 3′-UTR of Vtg mRNA, stabilizing its target through blocking its cleavage by mRNA endonucleases [24]. Assembly of Vtg [25] may be associated with protein disulfide isomerase, one of the most abundant proteins in the *N. vectensis* (Fig. 2B).

In oviparous eumetazoans, extra-ovarian and intra-ovarian synthesized Vtg are sequestered selectively into the oocyte by receptor-mediated endocytosis. Specific oocyte Vtg receptors are members of the low-density lipoprotein receptors (LDLR) [26]. We identified putative LDLRs (A7RXB8, A7RXB7) and a very-low-density lipoprotein receptor (VLDLR; A7RXU8) in the oocytes, suggesting an association with endocytosis of Vtg or lipid uptake. Interestingly, Trip10 (CIP4; A7RMS6), a player in endocytosis was identified in the *N. vectensis* oocyte, but not in the mouse. In vertebrates, Vtg is proteolytically cleaved by cathepsin D, after its uptake into the oocyte. A secondary cleavage may take place by cathepsins B and L [26]. We identified three putative lysosomal cathepsins functioning as

cysteine or aspartyl proteases in *N. vectensis* oocytes: cathepsin B (A7SNX0), cathepsin D (A7RH56) and cathepsin Z-like (A7SGN5); the first two were also found in the mouse oocyte.

Because lipoprotein receptors facilitate the uptake of lipids into cells, and since fatty acid metabolism is enriched in oocytes (see below), we searched for lipoproteins and proteins associated with lipid droplets that have been structurally identified within *N. vectensis* oocytes [17]. An apolipoprotein D (Apo D) (A7SH61), a member of the lipocalin protein family that binds small hydrophobic ligands was identified in *N. vectensis* oocytes [27]. In contrast, Apo A1 and Apo E, but not Apo D, were identified in the mouse oocyte. Perilipin-2 (A7SZB9), a protein specific to lipid droplet was identified in the *N. vectensis* oocyte and also several proteins involved in membrane trafficking such as Rabs (A7RMA6, A7ST02, A7RZ04, A7S806, A7SMT0, A7RER3, A7T2D5, A7RT62, A7RG79, A7S5I6) and a Snare protein (A7S1V0), all of which were shown to be associated with lipid droplets [28], but further studies are required to verify their function in *N. vectensis* oocytes. Flotillin-2, a marker of lipid rafts, was recently shown to be expressed in the mouse oocytes [29], and we also identified a Flotillin 2-like protein (A7SF43) in *N. vectensis* oocytes.

3.3. *N. vectensis oocyte contains envelop proteins similar to vertebrates*

Zona pellucida (ZP) domain-containing proteins are the main proteins in vertebrate egg coat [30]. We identified one protein (A7S0C8) with a zona pellucida domain containing the eight conserved cysteine residues and the putative furin cleavage site. Interestingly, there are 16 genes in the *N. vectensis* genome with the ZP domain, but only one protein was identified in the oocytes.

3.4. *Maternal and germ-cell specific proteins are conserved*

To identify additional *N. vectensis* oocyte-specific proteins we looked for germ cell specific proteins, and identified 11 proteins containing DEAD box helicases (Table S1), which participate in RNA metabolism. Orthologs of *vasa* are found throughout the animal kingdom and *vasa* is required for the efficient translation of *cyclinB* mRNA [31]. *Nvvas1*, *Nvvas2* and *Nvpl10* genes in *N. vectensis* were shown to be expressed in primordial germ cells (PGCs) and in somatic cells [18]. Interestingly, *Nvvas1*, but not *Nvvas2* or *Nvpl10*, was expressed in fertilized eggs. In the unfertilized egg we identified Vas2, Vas2-like (A7RNU5, A7T8H9) and Pl10 (A7SM49) proteins but were unable to detect Vas1 protein. However, Nos2 another known marker for oocytes and PGCs, that was shown to be expressed in fertilized eggs [18] was not detected, possibly because of its low abundance. A protein with high similarity to Dicer1 (A7RWA7) that has an essential function in completion of meiosis in mouse oocytes, was identified in *N. vectensis* [32]. We identified two Piwi proteins, containing the Piwi and the Paz domains in *N. vectensis* (A7RFC2; A7SQI3) and an additional partial Piwi protein (A7T755). Piwi proteins bind to Piwi-interacting RNAs (piRNAs), mediate epigenetic programming and posttranscriptional regulation and are active in germline specification, gametogenesis, stem-cell maintenance and transposon silencing. They contain multiple arginine repeats, which are potential methylation sites that may interact with different Tudor proteins [33]. Six proteins containing Tudor domains were identified (A7SMF2; A7SJJ01; A7RXU0; A7T738; A7SP53; A7RS72), three of them similar to Tdrd1/2/6 and one with high similarity to Tdrd11. These proteins bind Piwi proteins and serve different molecular functions. We identified three more RNP families that regulate post-transcriptional mechanisms and play important roles during gametogenesis and early embryonic development. Elav (Embryonic Lethal abnormal vision) proteins have been shown to control oogenesis and neuronal differentiation [34]. We identified NvElav1 protein in the *N. vectensis* oocyte (A7SL64; A7T0E1). *Elav1* mRNA had been reported in *N. vectensis* only at mid gastrulation in ectodermal cells that play a role in the emerging neural system [35,36]. We have also identified two other RNP proteins from the CUG-BP, Elav-like family (CELF), in the oocyte. CELF proteins promote rapid deadenylation and silencing of maternal transcripts and are mostly involved with cell cycle control and oocyte maturation [37]. Musashi (Msi) RNP are regulators of translation during germ cell development and are necessary for meiotic progression and correct chromosome segregation [38]. We identified three Msi proteins (A7SIF0; A7SSE1; A7T7K2) containing two conserved N-terminal tandem RNA recognition motifs. Interestingly, another *msi* transcript had been identified in the *N. vectensis* oral ectoderm at the planula stage [35].

Proteins of the LSm family including the Sm proteins were identified in mouse MII oocytes [10]. These proteins form a specific part of the small nuclear RNPs (snRNPs) and participate in the processing of pre-mRNAs to mature mRNAs. We identified 14 proteins (A7RTS4; A7SNR1; A7RM92; A7SSP8; A7SUU3; A7T8X0; A7RIQ2; A7RHB0; A7RX20; A7T1G2; A7RII4; A7RNM2; A7RYM0; A7SAT2) containing the LSm domain in *N. vectensis* oocytes, among them six LSm proteins (LSm1, 2, 3, 4, 6 and 8). The complex of LSm 2–8 in association with U6 small nuclear RNA (snRNA) is a component of the splicesome complexes in which pre-mRNA splicing occurs. Sm1–7 also function in degradation of mRNA by the deadenylation-dependent pathway of mRNA turnover. In mouse, granules at the cortex of the growing oocytes serve as an mRNA storage compartment. These granules, which are related to the P bodies, are important for early embryonic cell divisions. Their main proteins are Mater (NLRP5), Floped (OOEP), Padi6, Tle6 and Filia (2410004A20Rik) [32]. Null mutants of the first four proteins result in embryo arrest at the two-cell stage. Only Filia (A7RIJ4) was identified in the *N. vectensis* oocyte. This protein was suggested to play a role in spindle formation and a delay in embryo cell divisions and aneuploidy is seen in *filia*-null mice. Other essential maternal proteins located in the mouse oocyte cortex, as Zar1 and Tcl1a, were also missing from *N. vectensis* oocyte. However, Uchl1, (A7S784), which participates in the ubiquitin–proteasome pathway for protein degradation, was detected in the oocytes.

Substantial changes in chromatin configuration are needed for oocyte growth and genome decondensation immediately after fertilization. MII mouse oocytes display enrichment of epigenetic modification enzymes [13]. We identified three chromatin remodeling proteins homologous to Npm2 (A7RZK8), Hr6a (A7S6Q0), and Ssmarca5 (A7S667),

Table 1 – The most enriched pathways in *N. vectensis* oocytes.

Term	Oocyte	Ref[a]	FDR
Ribosome	67	72	0
Proteasome	36	39	1.15E−11
Valine, leucine and isoleucine degradation	34	38	3.20E−10
Citrate cycle (TCA cycle)	23	27	4.85E−06
Oxidative phosphorylation	49	77	1.33E−05
Propanoate metabolism	22	27	2.74E−05
Fatty acid metabolism	24	33	0.00027
Pyruvate metabolism	22	30	0.00043
Glycolysis/gluconeogenesis	22	31	0.00086
beta-Alanine metabolism	16	21	0.00203
Phagosome	35	60	0.00306
Aminoacyl-tRNA biosynthesis	17	24	0.00451
Pentose phosphate pathway	14	19	0.00706
Butanoate metabolism	13	18	0.01362
Glyoxylate and dicarboxylate metabolism	11	15	0.02401
Alanine, aspartate and glutamate metabolism	16	26	0.04356

[a] Ref: *N. vectensis* background.

all previously shown in mouse to be involved in oocyte and embryo development and to play a role in fertility [32].

3.5. *Cell cycle is suspended in ovulated oocytes at a specific stage of meiosis*

Cell-cycle arrest differs between species and its onset is highly regulated in oocytes [3]. We found that cell cycle constitutes 10% of the biological processes in *N. vectensis* oocytes (Fig. S1). We identified Cdk1 (A7RXS1), which interacts with cyclin B to form the complex M-phase promoting factor needed for G1/S and G2/M phase transitions. Several cyclins have been identified (A7SPG5; A7SFJ1; A7SD85), among them Cdc20 (A7SY11), which is essential during anaphase and surprisingly was not found in the mouse proteomics analyses [11,13].

3.6. *Oocyte regulatory pathways*

Reproductive processes in vertebrates and invertebrates are regulated by steroid hormones and vertebrate-type sex steroid hormones were identified in cnidarian tissues [39,40]. We identified estradiol 17-beta-dehydrogenase 12 (A7S4A6) in oocytes. This enzyme converts estrogen into estradiol in vertebrate ovarian tissue but can also function in fatty acid elongation and in biosynthesis of unsaturated fatty acids. We also identified five proteins associated with retinoid metabolism (A7RY06; A7S0P8; A7SJM9; A7SZ09; A7SP30). Retinoic acid affects pattern specification in hydroid polyps and neuronal differentiation in Anthozoa [41,42]. In higher organisms it participates in a broad range of physiological processes during embryogenesis. The 14-3-3 protein family, which mediates signal transduction by binding to phosphoserine-containing proteins, participates in a broad range of functions including cell cycle regulation, control of metabolism, lipid droplet function, apoptosis and control of gene transcription [43]. In the oocyte, members of this family were suggested to be active during meiosis and oocyte maturation. Mammals contain seven isoforms, all expressed in the oocyte, whereas *N. vectensis* was found to contain three relatively abundant isomers (beta, gamma and epsilon) (A7S0E0; A7S252; A7S4S2) (Table S1).

3.7. *Functional enriched KEGG pathways are shared between N. vectensis and mouse*

We found 16 pathways that are enriched in *N. vectensis* oocytes (Tables 1 and S3) and also in the mouse oocyte (Table S4). We could distinguish three main patterns by comparing the pathways in oocytes of *N. vectensis* and MII mouse. In Pattern 1, almost all of the proteins identified in *N. vectensis*, had corresponding proteins in the same pathway in the mouse oocyte (Fig. 4A). Pattern 2 shows numerous proteins common to both, but several additional proteins were specific either to the oocytes of *N. vectensis* or to those of mouse, indicative of the plasticity in the choice of metabolites in these pathways (Fig. 4B). In Pattern 3, we found several *N. vectensis* proteins within a pathway that we were unable to identify in the mouse oocyte, indicating that the activity of these proteins was more specific (Fig. 4C). Interestingly, in the lysosome pathway these latter proteins had glycosidase activity (Fig. 4C). More details on the enriched pathways in the *N. vectensis* oocyte are described below and summarized in Fig. 5.

3.7.1. *Translational machinery*

We found that about 67 of the 72 known proteins (Tables 1 and S3) were associated with ribosome structure in ovulated *N. vectensis* oocytes. Because mature oocytes are transcriptionally quiescent, the marked abundance of ribosomal proteins points to preparation for the translation of stored or newly synthesized mRNA in developmental stages post-fertilization [3]. The pathway of aminoacyl t-RNA biosynthesis is enriched in *N. vectensis* oocytes and those displayed were identical with those in the mouse oocyte. In addition, six ADP ribosylation factors involved in posttranslational modification were identified (A7RLK5; A7SVC4; A7S214; A7RJE1; A7RS05; A7RT65). These proteins play a role in regulation of asymmetric division in meiosis during oocyte maturation [13].

3.7.2. *Degradation of proteins*

The proteasome pathway is clearly displayed in *N. vectensis* (Tables 1 and S2), and we were able to detect an association between 17 proteins and the putative ubiquitin-mediated

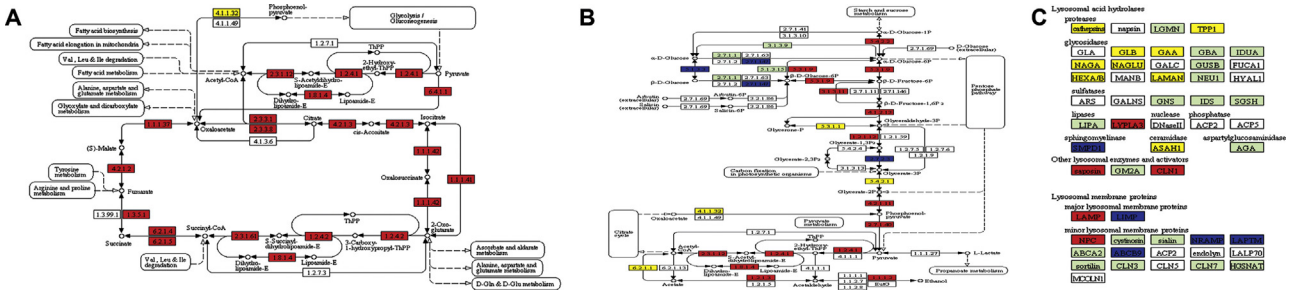

Fig. 4 – Three metabolic patterns of oocyte proteins associated with KEGG functional pathways. Pattern 1 is depicted for the citrate cycle (A) and a similar pattern was found for the following pathways: fatty acid synthesis, aminoacyl-tRNA biosynthesis, fatty acid metabolism, ribosome, proteasome, valine, leucine and isoleucine metabolism, oxidative phosphorylation. Pattern 2 is shown for glycolysis/gluconeogenesis (B) and similar patterns were displayed by the pathways for propanoate metabolism, pyruvate metabolism, beta-alanine metabolism, phagosome, pentose phosphate pathway, glyoxylate and dicarboxylate metabolism, alanine, asparate and glutamate metabolism. Pattern 3 is shown for proteins associated with the lysosome (C) and a similar pattern was found for glycosaminoglycan and glycan degradation. Proteins found only in *N. vectensis* are shown in yellow, proteins shared between *N. vectensis* and mouse oocyte in red and proteins identified only in the mouse oocyte in blue. Proteins in green are *N. vectensis* reference background proteins of the shown pathways that were not identified in the current study. (For interpretation of the references to color in text, the reader is referred to the web version of this article.)

proteolysis pathway. In addition to proteins that are damaged or misfolded, proteins are targeted for degradation during the oocyte-to-zygote transition stage.

3.7.3. *Energy metabolism*

Almost all of the enzymes associated with the citrate cycle pathway were identified in both *N. vectensis* and mouse oocytes (Fig. 4A), indicating the oxidation of acetate derived from amino acids and fatty acids. Amino acids can enter the citric cycle at different points and the generated NADH is fed into the oxidative phosphorylation pathway. The enriched pathways detected for amino acid metabolism as well as putative links with the citrate cycle, glycolysis, pyruvate metabolism and the pentose phosphate pathway point to an important role for amino acids in generating energy in the oocyte or in subsequent developmental stages. We identified proteins that were associated with the peroxisome pathway and function in lipid metabolism. Unlike the mouse oocyte, *N. vectensis* oocytes display in the peroxisome pathway an enzyme associated with α-oxidation (2-hydroxyacyl-CoA lyase 1, A7SFE8). A long-chain-fatty-acid – ligase protein (A7SW65) was specifically identified in *N. vectensis* oocytes. This protein is a member of the ligase family that activates the breakdown of complex fatty acids.

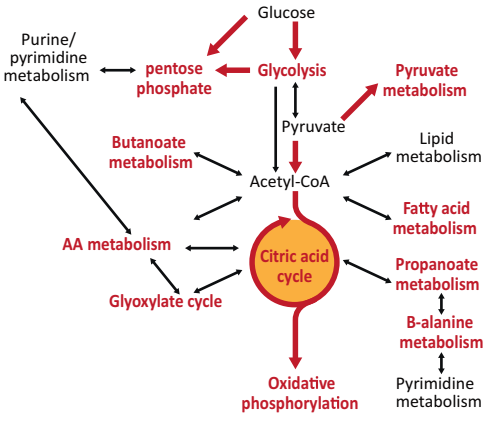

Fig. 5 – Schematic presentation of the metabolic pathways in *N. vectensis* oocyte. Enriched pathways are marked in red. (For interpretation of the references to color in text, the reader is referred to the web version of this article.)

3.7.4. *Fatty acid and lipid metabolism*

The enriched fatty acid metabolic pathway that we detected in *N. vectensis* oocytes leads to the formation of acetyl-CoA for yielding energy through the citrate cycle. It was also found here to be connected with alanine and aspartate metabolism.

3.7.5. *Carbohydrate metabolism*

We found that the pentose phosphate pathway in *N. vectensis* oocytes is enriched and facilitates the synthesis of 5-carbon sugars. We also found the ribose phosphate pyrophosphokinase P, suggesting a link with purine, pyrimidine and histidine metabolism (Fig. 5).

3.7.6. *Protection against oxidative stress*

Reactive oxygen species are produced by metabolic pathways, especially by oxidative phosphorylation, and therefore proteins with a role in antioxidant activity are important in maintaining oocyte quality. We found numerous proteins associated with antioxidant activity including superoxide dismutase [Cu-Zn] (A7S2P2), superoxide dismutase (SOD2; A7RHP7), glutathione peroxidase (A7RH42), peroxiredoxin 4 precursor (A7RK73), peroxiredoxin 6-like (A7RMT9), peroxiredoxin-mitochondrial-like (A7SG98), ferritin (A7S8I5, A7SM22, A7SLM6, A7S8I6) and catalase-like isoform 1 (A7S3Q8).

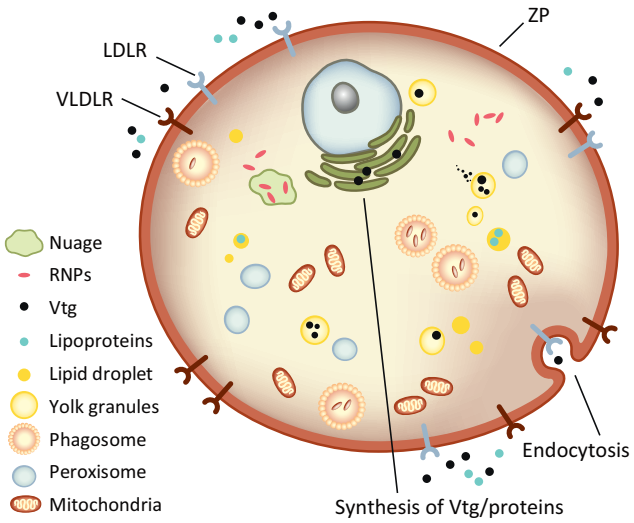

Fig. 6 – A model of the *N. vectensis* oocyte. Vtg can be synthesized in the oocyte but may also be transported into the oocyte via receptor-mediated endocytosis, involving a putative LDLR receptor. Subsequently, Vtg will accumulate in yolk granules together with cathepsins. The putative VLDLR may facilitate the uptake by the oocyte of lipids or lipophilic molecules that are deposited in lipid droplets. Oocyte specific RNP's may be associated with nuage or dispersed within the oocyte. The ZP protein may form part of the egg protein cover. Metabolic processes yielding energy take place in the peroxisome and mitochondria.

4. Discussion

The current study aims at identifying the proteome of mature ovulated eggs of a basal metazoan, the cnidarian *N. vectensis* and reveals for the first time proteins that were conserved through evolution.

A major difference between the oviparous *N. vectensis* and mammalian oocytes is in the abundance of vitellogenin (Fig. 2), the prominent constituent of yolk proteins in most eumetazoan oviparous eggs [26]. Yolk proteins are a predominant source of essential amino acids, phospholipids, cholesterol, phosphate and vitamins and are stored in yolk platelets in oviparous eggs, including *N. vectensis* [17]. Developing *N. vectensis* oocytes are surrounded by trophonemata, structures unique to cnidarians that play a role in the transport of extracellular digestive products from the gastrovascular cavity to the oocyte [17]. The site of Vtg synthesis in *N. vectensis* has yet to be established. However, recently, in a transcriptomics study of early development stages of *N. vectensis*, low amounts of Vtg mRNA were found in fertilized eggs [44]. That report, together with our finding of vigilin, an RNA-binding protein that stabilizes Vtg mRNA, suggests that Vtg is synthesized within the oocyte. Interestingly, in corals Vtg mRNA is expressed in mesenterial somatic cells but not in oocytes [45]. The presence of the lipoprotein receptors (LDLR and VLDLR) points to uptake of Vtg and/or lipoproteins from the trophocytes, in addition to intra-oocytic synthesis (Fig. 6). In the mouse, most of the metabolites are supplied by granulosa and cumulus cells surrounding the oocyte. A surprising finding was that all of the enriched KEGG functional pathways identified here in *N. vectensis* were also enriched in the mouse MII oocyte. A large number of the KEGG pathways are associated with metabolism of proteins, amino acids, fatty acids and metabolic pathways yielding ATP (Fig. 5). The prominence of metabolic pathways in oocytes was also demonstrated for *C. elegans* and for fish suggesting their importance for early embryo development [6,46]. Taken together, these observations indicate substantial conservation in oocyte functional pathways.

The proteomic profile of oocytes reveals an abundance of proteins associated with the translational machinery and cytoskeletal structure. Ovulated eggs are transcriptionally quiescent and major embryonic transcription is not initiated until the two cell stage in the mouse [12] and the blastula stage in *N. vectensis* [44]. Thus, the protein profile of *N. vectensis* oocytes reflects the well-prepared state of the oocyte for the onset of massive translation and transcription after fertilization. During oogenesis, numerous transcripts undergo post-transcriptional stabilization and are localized within protected RNP complexes, known as perinuclear granules, Balbiani bodies, sponge bodies, or nuage [47]. Nuage was identified in *N. vectensis* oocytes and may carry these RNPs [17]. An array of known protein components of RNP granules in animal oocytes was identified in the present study indicating their ancient origin. Germ plasm marker such as Vasa, Dicer, Piwi, and Sm proteins and proteins with Tudor domains were identified here. The distribution and localization of transcripts in distinct compartments are devices adopted by oocytes to rapidly and finely tune the translation of specific RNAs.

In vertebrates, the oocyte is surrounded by an envelope consisting of zona pellucida proteins that have different functions during oocyte maturation and fertilization. In invertebrates, zona pellucida proteins have been identified in various cells of worms and flies, and they play an important role in the developmental process [30]. Cnidaria zona pellucida proteins have been identified in the jellyfish *Aurelia aurita* in the mesoglea layer and in the anomalous pole of the oocytes [48]. In this study we found a zona pellucida protein with high similarity to the mouse ZP2. Additional studies are needed to demonstrate the localization of this protein to the oocyte envelopes.

Various proteins associated with regulatory pathways have been found in *N. vectensis*. One of these was the 14-3-3 protein, associated with binding to CDC25 in the G2 arrested *Xenopus* oocytes and an essential role in normal meiotic spindle formation during in vitro maturation of mouse oocytes [43]. In addition, proteins associated with retinoid and steroid metabolism were identified in oocytes but the presence of vertebrate type steroids in *N. vectensis* as well as endocrine regulation, remains an open question [39,40]

The present study contributes novel and important information on the proteins associated with developmental competence in eumetazoa eggs. These proteins facilitate the formation of pluripotent zygote with reprogramming capability and a viable germ line. Transcriptome profiling has shown that most genes expressed in oocytes are conserved among species [4,5]. Our study extends this general conclusion to the oocyte proteome by comparing the oocytes of two widely

diverse organisms, a basal eumetazoan and a mammalian species, thereby spanning ~500 million years of evolution. It suggests that the oocyte proteome template predates the divergence of the cnidarian and bilaterian lineages and raises a question on its origin.

5. Conclusions

This study provides an insight into the primordial proteome characteristics of a mature oocyte that facilitates the formation of a pluripotent zygote. We compared the proteome profiles of mature ovulated oocytes of the Cnidaria basal eumetazoan, the starlet sea anemone *N. vectensis*, with published data of mammalian mouse mature oocytes. We established the first catalog of cnidarian oocyte proteins containing 1837 proteins. Our findings reveal highly conserved ancient organization of life processes and expand our knowledge on the basal template of a mature oocyte that forms the basis for successful reproduction in eumetazoa.

Authors' contribution

T.L. and E.L. designed research; V.B. performed research; T.Z. performed proteomic analysis; T.L., V.C., M.M.G. and E.L. performed analysis; T.L., A.A., T.Z. and E.L. wrote the paper.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgments

We thank the Bioinformatics Core Facility at Ben-Gurion University and the Smoler Proteomics Center at the Technion for their assistance in the bioinformatics analysis and proteomic profiling. The work was supported by Ministry of Science and Technology (MOST).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.euprot.2014.01.003.

REFERENCES

[1] Barckmann B, Simonelig M. Control of maternal mRNA stability in germ cells and early embryos. Biochim Biophys Acta 2013;1829:714–24.
[2] Song JL, Wessel GM. How to make an egg: transcriptional regulation in oocytes. Differentiation 2005;73:1–17.
[3] Tadros W, Lipshitz HD. The maternal-to-zygotic transition: a play in two acts. Development 2009;136:3033–42.
[4] Evsikov AV, Graber JH, Brockman JM, Hampl A, Holbrook AE, Singh P, et al. Cracking the egg: molecular dynamics and evolutionary aspects of the transition from the fully grown oocyte to embryo. Genes Dev 2006;20:2713–27.
[5] Vallée M, Aiba K, Piao Y, Palin M-F, Ko MSH, Sirard M-A. Comparative analysis of oocyte transcript profiles reveals a high degree of conservation among species. Reproduction 2008;135:439–48.
[6] Chik JK, Schriemer DC, Childs SJ, McGhee JD. Proteome of the *Caenorhabditis elegans* oocyte. J Proteome Res 2011;10:2300–5.
[7] Roux MM, Townley IK, Raisch M, Reade A, Bradham C, Humphreys G, et al. A functional genomic and proteomic perspective of sea urchin calcium signaling and egg activation. Dev Biol 2006;300:416–33.
[8] Ziv T, Gattegno T, Chapovetsky V, Wolf H, Barnea E, Lubzens E, et al. Comparative proteomics of the developing fish (zebrafish and gilthead seabream) oocytes. Comp Biochem Phys D 2008;3:12–35.
[9] Knoll-Gellida A, Andre M, Gattegno T, Forgue J, Admon A, Babin P. Molecular phenotype of zebrafish ovarian follicle by serial analysis of gene expression and proteomic profiling, and comparison with the transcriptomes of other animals. BMC Genomics 2006;7:46.
[10] Ma M, Guo X, Wang F, Zhao C, Liu Z, Shi Z, et al. Protein expression profile of the mouse metaphase-II oocyte. J Proteome Res 2008;7:4821–30.
[11] Pfeiffer MJ, Siatkowski M, Paudel Y, Balbach ST, Baeumer N, Crosetto N, et al. Proteomic analysis of mouse oocytes reveals 28 candidate factors of the reprogrammome. J Proteome Res 2011;10:2140–53.
[12] Yurttas P, Morency E, Coonrod SA. Use of proteomics to identify highly abundant maternal factors that drive the egg-to-embryo transition. Reproduction 2010;139:809–23.
[13] Wang S, Kou Z, Jing Z, Zhang Y, Guo X, Dong M, et al. Proteome of mouse oocytes at different developmental stages. Proc Natl Acad Sci USA 2010;107:17639–44.
[14] de Sousa Abreu R, Penalva LO, Marcotte EM, Vogel C. Global signatures of protein and mRNA expression levels. Mol Biosyst 2009;5:1512–26.
[15] Technau U, Steele RE. Evolutionary crossroads in developmental biology: Cnidaria. Development 2011;138:1447–58.
[16] Putnam N, Srivastava M, Hellsten U, Dirks B, Chapman J, Salamov A, et al. Sea anemone genome reveals ancestral eumetazoan gene repertoire and genomic organization. Science 2007;317:86–94.
[17] Eckelbarger KJ, Hand C, Uhlinger KR. Ultrastructural features of the trophonema and oogenesis in the starlet sea anemone, *Nematostella vectensis* (Edwardsiidae). Invertebr Biol 2008;127:381–95.
[18] Extavour CG, Pang K, Matus DQ, Martindale MQ. *vasa* and *nanos* expression patterns in a sea anemone and the evolution of bilaterian germ cell specification mechanisms. Evol Dev 2005;7:201–15.
[19] Fritzenwanker J, Technau U. Induction of gametogenesis in the basal cnidarian *Nematostella vectensis* (Anthozoa). Dev Genes Evol 2002;212:99–103.
[20] Genikhovich G, Technau U. Induction of spawning in the Starlet Sea Anemone *Nematostella vectensis*, in vitro fertilization of gametes, and dejellying of zygotes. Cold Spring Harbor Protoc 2009, pdb.prot5281.
[21] Cox J, Mann M. MaxQuant enables high peptide identification rates, individualized p.p.b.-range mass accuracies and proteome-wide protein quantification. Nat Biotechnol 2008;26:1367–72.
[22] Xie C, Mao X, Huang J, Ding Y, Wu J, Dong S, et al. KOBAS 2.0: a web server for annotation and identification of enriched pathways and diseases. Nucleic Acids Res 2011;39:W316–22.
[23] Hayward A, Takahashi T, Bendena WG, Tobe SS, Hui JHL. Comparative genomic and phylogenetic analysis of vitellogenin and other large lipid transfer proteins in metazoans. FEBS Lett 2010;584:1273–8.

[24] Cunningham KS, Dodson RE, Nagel MA, Shapiro DJ, Schoenberg DR. Vigilin binding selectively inhibits cleavage of the vitellogenin mRNA 3′-untranslated region by the mRNA endonuclease polysomal ribonuclease 1. Proc Natl Acad Sci USA 2000;97:12498–502.
[25] Mann CJ, Anderson TA, Read J, Chester SA, Harrison GB, Köchl S, et al. The structure of vitellogenin provides a molecular model for the assembly and secretion of atherogenic lipoproteins. J Mol Biol 1999;285:391–408.
[26] Babin P, Carnevali O, Lubzens E, Schneider W. Molecular aspects of oocyte vitellogenesis in fish. In: Babin P, Cerdà J, Lubzens E, editors. The fish oocyte. The Netherlands: Springer; 2007. p. 39–76.
[27] Yao Y, Vieira A. Comparative 17β-estradiol response and lipoprotein interactions of an avian apolipoprotein. Gen Comp Endocrinol 2002;127:89–93.
[28] Yang L, Ding Y, Chen Y, Zhang S, Huo C, Wang Y, et al. The proteomics of lipid droplets: structure, dynamics, and functions of the organelle conserved from bacteria to humans. J Lipid Res 2012;53:1245–53.
[29] Buschiazzo J, Ialy-Radio C, Auer J, Wolf J-P, Serres C, Lefèvre B, et al. Cholesterol depletion disorganizes oocyte membrane rafts altering mouse fertilization. PLoS ONE 2013;8:e62919.
[30] Plaza S, Chanut-Delalande H, Fernandes I, Wassarman PM, Payre F. From A to Z: apical structures and zona pellucida-domain proteins. Trends Cell Biol 2010;20:524–32.
[31] Yajima M, Wessel GM. The multiple hats of Vasa: its functions in the germline and in cell cycle progression. Mol Reprod Dev 2011;78:861–7.
[32] Li L, Zheng P, Dean J. Maternal control of early mouse development. Development 2010;137:859–70.
[33] Juliano C, Wang J, Lin H. Uniting germline and stem cells: the function of Piwi proteins and the piRNA pathway in diverse organisms. Annu Rev Genet 2011;45:447–69.
[34] Colombrita C, Silani V, Ratti A. ELAV proteins along evolution: back to the nucleus? Mol Cell Neurosci 2013;56:447–55.
[35] Marlow HQ, Srivastava M, Matus DQ, Rokhsar D, Martindale MQ. Anatomy and development of the nervous system of *Nematostella vectensis*, an anthozoan cnidarian. Dev Neurobiol 2009;69:235–54.
[36] Nakanishi N, Renfer E, Technau U, Rentzsch F. Nervous systems of the sea anemone *Nematostella vectensis* are generated by ectoderm and endoderm and shaped by distinct mechanisms. Development 2012;139:347–57.
[37] Dasgupta T, Ladd AN. The importance of CELF control: molecular and biological roles of the CUG-BP, Elav-like family of RNA-binding proteins. Wiley Interdiscip Rev RNA 2012;3:104–21.
[38] Gunter KM, McLaughlin EA. Translational control in germ cell development: a role for the RNA-binding proteins Musashi-1 and Musashi-2. IUBMB Life 2011;63:678–85.
[39] Markov GV, Tavares R, Dauphin-Villemant C, Demeneix BA, Baker ME, Laudet V. Independent elaboration of steroid hormone signaling pathways in metazoans. Proc Natl Acad Sci USA 2009;106:11913–8.
[40] Tarrant AM, Reitzel AM, Blomquist CH, Haller F, Tokarz J, Adamski J. Steroid metabolism in cnidarians: insights from *Nematostella vectensis*. Mol Cell Endocrinol 2009;301:27–36.
[41] Bouzaiene M, Angers A, Anctil M. Immunohistochemical localization of a retinoic acid-like receptor in nerve cells of two colonial anthozoans (Cnidaria). Tissue Cell 2007;39:123–30.
[42] Muller WA. Retinoids and pattern formation in a hydroid. J Embryol Exp Morphol 1984;81:253–71.
[43] De S, Marcinkiewicz J, Vijayaraghavan S, Kline D. Expression of 14-3-3 protein isoforms in mouse oocytes, eggs and ovarian follicular development. BMC Res Notes 2012;5:57.
[44] Helm R, Siebert S, Tulin S, Smith J, Dunn C. Characterization of differential transcript abundance through time during *Nematostella vectensis* development. BMC Genomics 2013;14:266.
[45] Shikina S, Chen C-J, Chung Y-J, Shao Z-F, Liou J-Y, Tseng H-P, et al. Yolk formation in a stony coral *Euphyllia ancora* (Cnidaria, Anthozoa): insight into the evolution of vitellogenesis in non-bilaterian animals. Endocrinology 2013;154:3447–59.
[46] Finn RN, Fyhn HJ. Requirement for amino acids in ontogeny of fish. Aquacult Res 2010;41:684–716.
[47] Voronina E, Seydoux G, Sassone-Corsi P, Nagamori I. RNA granules in germ cells. Cold Spring Harbor Perspect Biol 2011:3.
[48] Adonin L, Podgornaya O, Shaposhnikova T. Morphodynamics of the contract plate in the course of oocyte maturation in the scyphozoan *Aurelia aurita* (Cnidaria: Semaeostomae). Russ J Dev Biol 2012;43:17–24.